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Heterotrophic Plate Counts of Surface Water Samples by Using Impedance Methods

PETER A. NOBLE,[1,2] EDIE ASHTON,[2] CHARLES A. DAVIDSON,[2] and WILLIAM L. ALBRITTON[2]*

Department of Applied Microbiology and Food Science, University of Saskatchewan, Saskatoon, Saskatchewan S7K 0W0,[1] and Division of Environmental Microbiology, Provincial Laboratory of Public Health for Northern Alberta, University of Alberta, Edmonton, Alberta T6G 2J2,[2] Canada

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Membrane filtration, spread plating, and pour plating are conventional methods used to determine the heterotrophic plate counts of water samples. Impedance methods were investigated as an alternative to conventional methods, since sample dilution is not required and the bacterial count can be estimated within 24 h. Comparisons of impedance signals obtained with different water samples revealed that capacitance produced faster detection times than conductance. Moreover, the correlation between heterotrophic plate count and detection time was highest (r = 0.966) when capacitance was used. Linear and quadratic regressions of heterotrophic plate count and impedance detection time were affected by incubation temperatures. Regressions between heterotrophic plate counts based on conventional methods and detection times of water samples incubated at ≤25°C had R^2 values of 0.878 to 0.933. However, regressions using detection times of water samples incubated at ≥30°C had lower R^2 values, even though water samples produced faster detection times. Comparisons between broth-based versions of R2A medium and plate count agar revealed that the latter correlated highly with heterotrophic plate count, provided that water samples were incubated at 25°C and impedance measurements were conducted with the capacitance signal (r = 0.966). When the linear regression of this relationship was tested with 100 water samples, the correlation between predicted and actual $\log_{10}$ CFU milliliter^{-1} was 0.869. These results indicate that impedance methods provide a suitable alternative to conventional methods.

Heterotrophic plate counting (HPC) is a procedure used by public health laboratories to estimate the bacterial loads of surface water samples and to monitor the efficiency of disinfectants at water treatment plants. The number of heterotrophic bacteria in a water sample can be estimated from the CFU on a recognized agar-based medium after a specific incubation temperature and time. For example, when HPC is determined by using R2A medium, an incubation temperature of 20 or 28°C for 5 to 7 days is required. Incubation time can be reduced to 48 h when plate count agar (PCA) is used; however, the incubation temperature must be increased to 35°C (1). Comparison of these media by using water samples showed that PCA yields significantly lower counts and fewer pigmented bacterial colonies than does R2A (6, 7). These differences have been attributed to the low nutrient content of R2A and the lower incubation temperatures used for this medium. Longer incubation time is required for R2A, since bacterial colonies fail to reach a detectable size within 48 h.

Conventional techniques for HPC determination include membrane filtration, spread plating, and pour plating (1). A major drawback of these techniques is that water samples must be serially diluted to ensure that bacterial colonies are within a countable range (e.g., 30 to 300 colonies per plate). Serial dilutions increase the number of plates and media to be utilized and the number of water samples to be diluted by severalfold. Moreover, diluting and plating protocols increase inherent errors associated with the actual bacterial count.

Impedance methods offer an attractive alternative to conventional methods because there is no need to dilute water samples and because the bacterial count can be determined within 24 h. The principle of this technology is that subtle changes in the ionic composition of the medium affect the electrical conductance and capacitance (8). As bacteria metabolize, uncharged or weakly charged substrates are transformed into charged end products. Accumulation of these products increases the conductance of the medium and the capacitance at the electrode-medium interface. The amount of time required to cause a series of significant deviations from baseline impedance values is referred to as the detection time (DT), and this corresponds to a bacterial concentration of approximately 10^7 cells/ml (4). Since DT is inversely proportional to the bacterial concentration at the time of inoculation, this measure can be used to estimate the HPCs of water samples.

The focus of this study was to investigate whether impedance methods could be used to estimate the HPCs of surface water samples. We examined the effects of incubation temperature, impedance signal, sample size, and growth medium on HPC as a function of the DT. Using surface water samples, we compared HPCs obtained by conventional methods with predicted HPCs obtained by impedance methods.

MATERIALS AND METHODS

Media. Plate count agar (PCA) (BBL Standard Methods Agar; Becton Dickinson Microbiology Systems, Cockeysville, Md.) was prepared by following the manufacturer's specifications. PCA-based broth (PCB) consisted of 5 g of tryptone, 2.5 g of yeast extract, and 1 g of glucose per liter. R2A plates were made by following the guidelines of Reasoner and Geldreich (7). R2A-based broth (R2B) was composed of R2A minus the agar component.

* Corresponding author.

Unless otherwise specified, both PCB and R2B were prepared at 4× concentration, autoclaved, and dispensed into 2.5-ml module wells to a volume of 0.33 ml. Unit concentration was obtained with the addition of 1 ml of water sample.

Water samples. Surface water samples (200 ml) were collected from different municipalities by public health agencies between the months of September 1990 and April 1991. Each 250-ml sterile polypropylene collection bottle contained a tablet consisting of 10 mg of sodium thiosulfate, 138 mg of sodium chloride, and 2 mg of Carbowax (Systems Plus, New Hamburg, Ontario, Canada). Water samples were received by the laboratory within 24 h of collection and were processed by following standard methods (1).

Impedance equipment. Impedance was measured by using the Bactometer (Vitek, Hazelwood, Mo.). This system consists of a computer terminal, printer, and two temperature-controlled incubators. Modules, which are designed to clip into an incubator, contained 16 wells; each well had two electrodes. At timed intervals, impedance measurements are sent from the electrodes to the computer terminal. The ability to detect significant deviations in the impedance signal was controlled by the algorithm or test type selected. Different test types permit the user to select the desired impedance signal, i.e., capacitance, conductance, or a combination of these signals, referred to as total impedance.

Experimental procedures. (i) Effects of incubation temperatures, impedance signal, and sample size on DT. Wells containing PCB were inoculated with 1 ml of water sample (in duplicate) and incubated at 20, 25, 30, or 35°C for 24 h. Total impedance, conductance, and capacitance was used to determine the DT. Wells containing PCB were inoculated (in duplicate) with 0.2, 1.0, or 2.0 ml of the water sample to give a final unit concentration and incubated at 25°C for 24 h.

(ii) Comparison of effect of impedance medium on DT. Module wells containing a final unit concentration of R2B or PCB were inoculated with 1.0 ml of water sample (in duplicate) and incubated at 25 or 35°C for 24 to 48 h. HPC for water samples was determined (in duplicate) by the spread plate method. Unless otherwise specified, R2A and PCA plates were incubated at 25°C for 168 h and 35°C for 48 h, respectively (1).

(iii) Linear and quadratic regressions between HPC and DT. The relationship between HPC and DT for 152 water samples was evaluated by using different impedance signals and incubation temperatures.

Data analysis. Linear and quadratic relationships between HPC and DT, analysis of variance, Duncan's multiple range test, and correlation coefficients (Pearson product-moment) were determined by using the Statistical Analysis System (SAS Institute Inc., Cary, N.C.).

RESULTS

Effects of incubation temperature, impedance signal, and sample size on DT. Incubation temperatures significantly influenced the DTs of water samples inoculated in PCB ($P < 0.0001$). Water samples incubated at low temperatures had longer DTs than samples incubated at high temperatures, suggesting that growth of heterotrophic bacteria in this medium is highly temperature dependent (Table 1). Although all 152 water samples had DTs when incubated at 25, 30, and 35°C, 12 samples did not have DTs when incubated at 20°C, likely due to insufficient growth within the allotted 24-h incubation period.

Comparison of the DTs between impedance signals showed that capacitance resulted in shorter DTs than did conductance and total impedance, regardless of incubation temperature. This result may be attributed to differences between impedance signals.

TABLE 1. DTs for water samples as a function of incubation temperature and impedance signal

Incubation temp (°C)	Mean DT (h) ± SD (n)[a] obtained by using:		
	Total impedance	Conductance	Capacitance
20	15.1 ± 4.8 (148)	15.3 ± 4.8 (140)	14.0 ± 4.4 (150)
25	10.7 ± 3.6 (152)	11.3 ± 3.9 (152)	9.8 ± 3.3 (152)
30	7.2 ± 2.3 (152)	7.5 ± 2.5 (152)	6.8 ± 2.2 (152)
35	6.2 ± 2.0 (152)	6.4 ± 2.1 (152)	6.1 ± 1.9 (152)

[a] n, number of samples inoculated in PCB.

Although 1.0- and 2.0-ml water samples had shorter DTs than did 0.2-ml samples for both total impedance and capacitance signals, this was not observed for the conductance signal (Table 2). For subsequent experiments, 1-ml water samples were used to inoculate module wells.

Comparison between HPCs and DTs for different media. Water samples inoculated in PCB appeared to have higher correlations with the HPC than did samples inoculated in R2B (Table 3). Although comparison of correlation coefficients between R2B and PCB by using the capacitance signal revealed that the latter was more highly correlated with HPC than the former, R2B had higher correlations with HPC than did PCB when the conductance signal was used. High correlations between PCB and HPC appear to be a function of the medium used for HPC determination. In general, when HPC was determined with R2A, low correlation coefficients were attained, despite incubation temperatures of 25 or 35°C (Table 3) and incubation times of 48 and 168 h. Similarly, increasing or decreasing the incubation temperatures of R2B cultures did not improve the correlations, regardless of the medium or incubation conditions used for HPC determination. Initial studies that examined the effects of low incubation temperatures with R2B revealed that most water samples did not have DTs even after 48 h of growth (data not shown).

Correlation coefficients between DTs of water samples inoculated in PCB and incubated at different temperatures revealed temperature-dependent relationships (Table 4). High correlations were found between adjacent incubation temperatures: 20 and 25, 30 and 35, and 25 and 30°C, respectively. The lowest correlation was between DTs of water samples incubated at 20 and 35°C, indicating that changes in DT at these temperatures may not be a function

TABLE 2. DTs for water samples as a function of sample size and impedance signal[a]

Sample size (ml)	n	Mean DT (h) ± SD obtained by using:		
		Total impedance[b]	Conductance	Capacitance[b]
0.2	30	17.3 ± 7.7	17.0 ± 7.4	16.2 ± 7.2
1.0	29	14.5 ± 6.1	15.0 ± 6.5	14.3 ± 6.7
2.0	30	14.9 ± 6.4	16.4 ± 8.0	14.1 ± 5.7

[a] Water samples were inoculated in PCB and incubated at 25°C.

[b] Significant difference ($P < 0.01$ for total impedance and $P < 0.001$ for capacitance) as determined by analysis of variance; no significant difference between 1.0- and 2.0-ml sample sizes, as determined by Duncan's multiple range test ($\alpha = 0.01$).

TABLE 3. Correlation coefficients of DTs determined by the impedance method and HPCs determined by the spread plate methods for 31 water samples

Impedance medium	Incubation temp (°C)	Impedance signal	r with HPCs determined with:		
			PCA (35°C, 48-h incubation)	R2A (25°C, 168-h incubation)	R2A (35°C, 168-h incubation)
PCB	25	Conductance	−0.722	−0.631	−0.464
		Capacitance	−0.884	−0.823	−0.716
R2B	25	Conductance	−0.798	−0.725	−0.661
		Capacitance	−0.740	−0.737	−0.585

of slower metabolic activity but rather may be a function of changes in the bacterial composition.

Linear and quadratic regressions between HPC and DT. Incubation temperatures affected the regressions between HPC and DT regardless of impedance signal (Table 5). In general, ≤25°C incubation temperatures had higher R^2 values than did ≥30°C temperatures. Linear and quadratic regressions based on capacitance DTs consistently had higher R^2 values than did those based on conductance DTs, with the highest R^2 value occurring with water samples incubated at 25°C.

Comparison of the regression model equations showed that quadratic equations better explained the relationship between HPC and capacitance DT than did linear equations when incubation temperatures of ≥30°C were used (Table 5). However, this distinction between quadratic and linear model equations with capacitance DT became less significant at low incubation temperatures. Comparisons of model regressions between HPC and conductance DT at different incubation temperatures revealed that quadratic equations better explained these relationships than did linear equations, independently of temperature examined.

When the impedance signals of 152 samples incubated at 25°C were compared with HPCs obtained with capacitance, conductance, and total impedance, distinct differences in the dispersion of data was evident (Fig. 1). When the linear model equation for samples inoculated into PCB and incubated at 25°C were tested with 100 water samples by using the capacitance signal, the correlation between predicted and actual $\log_{10}$ CFU milliliter^{-1} was 0.869 (Fig. 2).

DISCUSSION

Although impedance methods have been used to predict the shelf lives of food products such as pasteurized milk (2), these methods have not been used to estimate the bacterial loads of water samples. In this study, impedance methods were adapted and compared with methods currently approved for HPC by the American Public Health Association (1). Agar-based media were not used for impedance measurements because of difficulties encountered when dispensing media into module wells. Instead, broth-based versions of R2A and PCA were conveniently dispensed into module wells immediately prior to inoculation with water samples. Of the several media approved for HPC, R2A was selected for this study because it promotes the growth of slowly growing and pigmented bacteria (7). PCA and PCB were also used in this study because these media recover rapidly growing heterotrophic bacteria.

Studies on the effects of incubation temperature, impedance signal, and sample size on DT indicate that fast DTs were obtained when (i) samples were incubated at ≥25°C, (ii) capacitance was used as the impedance signal, and (iii) ≥1-ml sample sizes were used as inocula. Since impedance is a function of the dynamic metabolic activity of bacteria (3), it is not surprising that incubation temperature affects DT. In this study, water samples that failed to produce impedance DTs when incubated at 20°C did so when incubated at ≥25°C, suggesting that bacterial growth within the allotted 24-h incubation time was insufficient to affect baseline impedance values. Since one of the goals of this study was to define an impedance method that could estimate HPC within 24 h, the 20°C incubation temperature was deemed unsuitable for further study.

Total impedance is a function of both conductance and capacitance, and differences between these signals have a combined effect on the total impedance DT. These signals differ in their responses to biochemical changes in the medium and at the electrode-medium interfaces. For example, conductance is sensitive to the accumulation and mobilization of ionized end products in the incubation medium, whereas capacitance is mainly affected by changes in pH and temperature (5). Biochemical changes which occur during the incubation account for different impedance DTs. Since in most cases, DTs occur earlier with the capacitance signal than with the conductance signal, one can assume that changes in pH occur prior to the significant accumulation of ionized metabolites. Preliminary studies with surface water samples indicated that subtle changes in the pH of the growth medium occurred concomitantly with capacitance DT (data not shown). Additional studies are needed to clarify differences between impedance signals.

Previous studies have shown that the relationship between $\log_{10}$ CFU milliliter^{-1} and DT becomes nonlinear when either high or low concentrations of organisms are encountered (4). This study found that the relationship between $\log_{10}$ CFU milliliter^{-1} and capacitance DT was linear between 10^1 to 10^7 CFU milliliter^{-1}, providing that incubation temperatures of ≤25°C were used. The high and low bacterial concentrations that were tested by our model equations were based on the bacterial counts obtained by conventional techniques. The lower limit of detection for HPC using PCA is 10 CFU milliliter^{-1}. HPCs lower than this limit obtained by either conventional methods or the impedance method

TABLE 4. Correlation coefficients of DTs obtained by using capacitance compared by incubation temperature ($P < 0.001$)

Incubation temp (°C)	r (no. of samples) with incubation temp (°C) of:		
	20	25	30
25	0.934 (150)		
30	0.798 (150)	0.900 (152)	
35	0.679 (150)	0.797 (152)	0.921 (152)

TABLE 5. Linear and quadratic models for the regression between HPC and DT as a function of incubation temperature and impedance signal

Incubation temp (°C)	Impedance signal	n	R^2 value	Model equation
20	Conductance	138	0.757[a]	$\log_{10}$ CFU ml^{-1} = (−0.201 × DT) + 7.165
			0.763[b]	$\log_{10}$ CFU ml^{-1} = (0.006 × DT^2) − (0.390 × DT) + 8.470
	Capacitance	148	0.878[a]	$\log_{10}$ CFU ml^{-1} = (−0.248 × DT) + 7.431
			0.879[b]	$\log_{10}$ CFU ml^{-1} = (0.001 × DT^2) − (0.285 × DT) + 7.665
25	Conductance	150	0.797[a]	$\log_{10}$ CFU ml^{-1} = (−0.267 × DT) + 6.970
			0.802[b]	$\log_{10}$ CFU ml^{-1} = (0.008 × DT^2) − (0.096 × DT) + 6.167
	Capacitance	150	0.932[a]	$\log_{10}$ CFU ml^{-1} = (−0.340 × DT) + 7.291
			0.933[b]	$\log_{10}$ CFU ml^{-1} = (0.004 × DT^2) − (0.267 × DT) + 6.972
30	Conductance	150	0.606[a]	$\log_{10}$ CFU ml^{-1} = (−0.357 × DT) + 6.650
			0.648[b]	$\log_{10}$ CFU ml^{-1} = (0.036 × DT^2) − (0.909 × DT) + 8.499
	Capacitance	150	0.696[a]	$\log_{10}$ CFU ml^{-1} = (−0.443 × DT) + 6.978
			0.729[b]	$\log_{10}$ CFU ml^{-1} = (0.050 × DT^2) − (1.113 × DT) + 8.984
35	Conductance	150	0.526[a]	$\log_{10}$ CFU ml^{-1} = (−0.398 × DT) + 6.489
			0.574[b]	$\log_{10}$ CFU ml^{-1} = (0.035 × DT^2) − (0.883 × DT) + 7.987
	Capacitance	150	0.529[a]	$\log_{10}$ CFU ml^{-1} = (−0.450 × DT) + 6.680
			0.584[b]	$\log_{10}$ CFU ml^{-1} = (0.046 × DT^2) − (1.059 × DT) + 8.519

[a] Linear.
[b] Quadratic.

may not be valid. On the basis of our model equation using the capacitance DT and an incubation temperature of 25°C, the maximum CFU milliliter^{-1} detectable by our method is ca. 10^7.

We found that 1.0- and 2.0-ml water samples produced faster capacitance DTs than did 0.2-ml samples. However, this difference was not apparent when conductance was used. Our findings are contrary to those of Silverman and Munoz (9), who found that large sample sizes lengthened the time required to produce conductance DTs. Although definite comparisons between their study and ours are complicated by such factors as medium, impedance signal, and electrode design, their study used much larger sample sizes (i.e., 20 ml) than ours. Differences in the time required to heat the 20-ml water sample to 44.5°C may account for the longer DTs observed in their study. For HPC determinations, large sample volumes are desirable simply because they allow the growth of a broader range of organisms than do smaller volumes. Unfortunately, the maximum volume permitted in the module wells used in this study is approximately 2.5 ml.

Comparisons between impedance media demonstrated that PCB provides a higher correlation with HPC than does R2B. As previously mentioned, PCB allows the growth of rapidly growing bacteria, while R2A permits the growth of slowly growing and pigmented bacteria. It is clear from the correlation between impedance signal and HPC with R2A that impedance methods used with PCB do not take into account heterotrophic bacteria that cannot grow on PCA. Even when R2B was incubated for 48 h, no or poor correlations were obtained regardless of the conventional method used to determine HPC. One limitation of the impedance method is that the estimated HPC based on DT corresponds to the HPC determined by conventional methods using PCA incubated at 35°C for 48 h.

It is important to recognize that fast DTs do not necessarily imply better correlations between HPC and DT. This study showed that low incubation temperatures provided higher R^2 values between HPC and DT than did high temperatures. Low R^2 values are most likely attributable to differences in the generation times of psychrotrophic and mesophilic bacteria. For example, at 37°C, mesophilic bacteria such as *Enterobacter cloacae* ATCC 23355 have generation times of 71 min. However, when this organism is grown at 35°C, there is a concomitant reduction in generation time to 49 min (4). This organism, along with many other mesophilic bacteria, reduces its generation time when grown under suboptimal conditions. In mixed cultures (such as heterotrophic bacteria found in water samples), low incubation temperatures prevent the rapid generation of mesophilic bacteria while providing better growth conditions for psychrotrophic bacteria. One could assume that at higher incubation temperatures, DTs are mostly determined by rapidly growing mesophilic bacteria rather than by psychrotrophic bacteria.

Although no studies have used capacitance to monitor bacterial growth, this signal has been used to measure the growth of yeasts such as *Saccharomyces cerevisiae*, *Candida utilis*, and *Kloeckera apiculata* (5). For these organisms, capacitance has been preferred over conductance because the former is 20 to 60 times more sensitive to their growth on tryptic soy broth or yeast carbon broth. One reason capacitance has not been used to monitor bacterial growth is because this signal was thought to be insensitive and subject to random fluctuations (8). However, Firstenberg-Eden and Zindulis (5) reported that changes in capacitance due to growth of *Escherichia coli* were not random. Furthermore, they recommended that conductance be used to detect bacterial metabolism when bacteria are grown in low-conductivity media such as PCB but that capacitance be used in high-conductivity media such as tryptic soy broth or brain heart infusion broth, because conductance is insensitive to metabolic changes in these media. Unfortunately, their recommendations are based on experiments with pure strains of *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, and *Streptococcus pyogenes*. No studies have investigated the use of impedance signals with mixed bacterial populations from environmental sources.

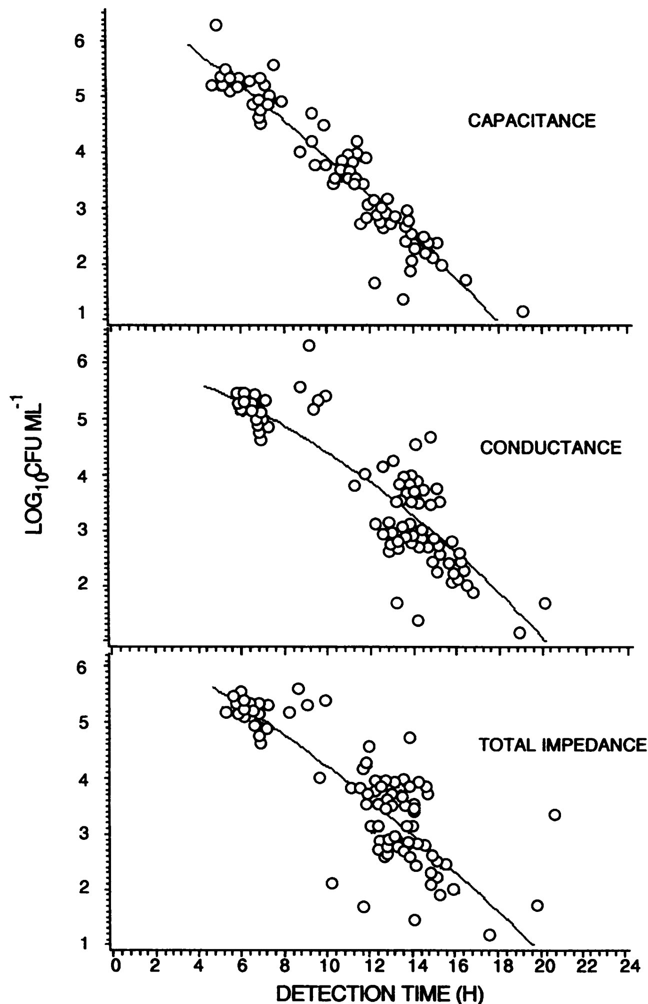

FIG. 1. Quadratic regressions of HPC and DT by impedance signal. Water samples were inoculated in PCB and incubated at 25°C. HPC was determined by the spread plate method by using PCA incubated at 35°C for 48 h.

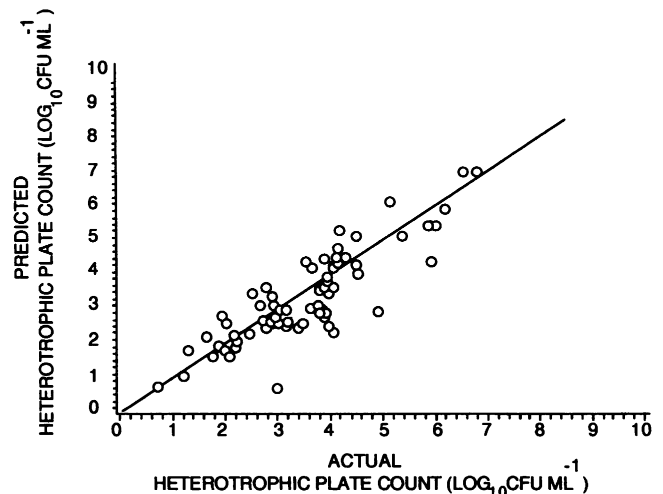

FIG. 2. Comparison of predicted HPCs based on DT, using the equation $\log_{10}$ CFU milliliter^{-1} = $(-0.340 \times DT) + 7.291$ and actual HPCs based on the spread plate method. DT was determined by using the capacitance signal with water samples inoculated in PCB and incubated at 25°C.

On the basis of our analysis of 152 water samples, we recommend the use of capacitance rather than conductance or total impedance simply because this signal is highly correlated with HPC. Contrary to the Richards et al. (8) study, we observed more scatter about the regression between HPC and DT when conductance and total impedance signals were used. Scatter about these regressions is likely caused by delayed or premature DTs. The absence or reduction of scatter in the regressions obtained for capacitance DT may be attributed to distinct and subtle changes in the pH of the medium close to the electrodes (5).

In summary, we found that impedance methods provided a suitable alternative to HPC determination by conventional methods, provided that 1-ml water samples were inoculated into PCB, incubated at 25°C, and monitored by the capacitance signal.

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